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The Automatic Determination of Trypsin Activity*)

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In the course of a study on the fractionation of crystallized trypsin the need of a method for the automatic determination of trypsin activity was felt.

Two such methods were developed employing the Technicon Auto Analyser system, one for the measurement of trypsin amidasic activity and another for the esterasic activity.

The use of benzoyl-L-arginine amide (BAA) [1, 2] as a substrate for the measurement of the amidasic activity of trypsin is well known. This substrate is hydrolyzed by trypsin to N-benzoyl-L-arginine and ammonia which in the present method is determined by using the alkaline phenol-hypochlorite reagent, as described by RUSSEL [3] and adapted to the Auto Analyzer system by FERRARI [4] and LOGSDON [5].

The determination of trypsin activity by using p-toluene sulfonyl-L-arginine methyl ester (TAME) as a substrate [6, 7] is an adaptation to the Auto Analyzer system of the method developed by SIEGELMAN AND COLABORATORS [8]. In this method methanol formed by tryptic hydrolysis of TAME is oxidized to formaldehyde by permanganate oxidation which is then measured by the chromotropic acid reaction.

METHODS

The trypsin samples used in this work were obtained from the *Worthington Biochemical Corporation* (Cryst. Trypsin, lyophilized, lot TL 747–48) Freehold, N. Jersey, USA and from *Novo Industri* (Cryst. Trypsin Novo, batch 114–3) Copenhagen, Denmark. The enzyme was dissolved in 0,01 N HCl and the actual trypsin concentration was obtained by measuring the absorbance at 280 mμ and using as extinction coefficient $E^{1\%}_{1cm} = 14.4$ [9].

A solution containing 500 μg/ml of trypsin was initially prepared and then diluted with 0.01N HCl to give the required concentrations.

DETERMINATION OF THE AMIDASIC ACTIVITY

Reagents: Benzoyl-L-arginine amide.HCl.H_2O (obtained from the *California Corporation for Biochemical Research*, Los Angeles, U.S.A.) was dissolved in 0.1N

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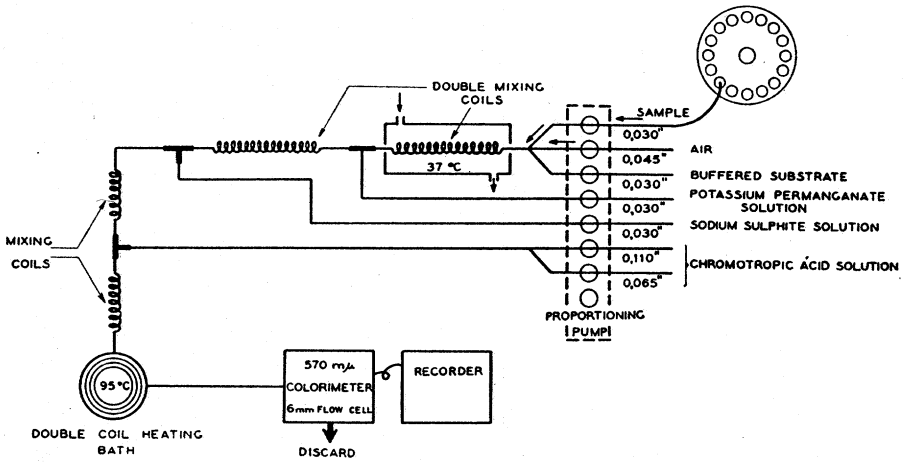

Fig. 2 — Flow chart and manifold components for the determination of esterasic activity.

For better results we found out that it is necessary to intercalate in the sampler plate a cup with N/100 HCl after each cup containing trypsin solution to be estimated. The sampler is set to function at 60 samples per hour resulting, therefore, in a rate of 30 determinations per hour.

RESULTS AND DISCUSSION

Figure 3 shows that the proportionality between concentration of the enzyme and the colour measured, i.e., the amount of ammonia liberated from BAA is good. A straight line is obtained for concentrations of crystallized trypsin from about 5 μg to 150 μg. Greater sensitivity can be achieved, however, by using a 0,040" manifold tubing for sampling and eliminating the 0,030" neutralizer tubing. This modification will double the sensitivity of the method and can be used whenever the buffer in which the BAA is dissolved is sufficient to control the pH of the mixture to be incubated with trypsin.

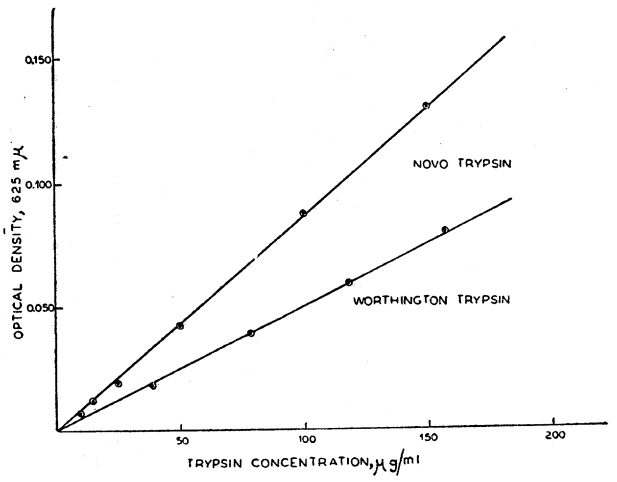

Fig. 3 — Calibration curve for trypsin amidasic activity.

Figure 4 shows the results of a study of the effect of BAA concentration on the reaction velocity using the present method. These data were obtained simply by connecting the 0,056" tubing to the sampler while the 0,030" tubing (previously connected to the sampler) was used to aspirate trypsin solution continuously. Buffered substrate solutions were put in the sampler plate in groups of four cups for each concentration studied, each group separated by three cups containing buffer only. The results of these experiments show that in the conditions used a zero order reaction is obtained only with a BAA concentration greater than 6×10^{-3}M. It is clear that greater sensitivity and better overall results should be obtained by using a BAA

Borate/HCl buffer of pH 8, to give the required concentration. In all determinations of trypsin specific activity a 2mM concentration of the substrate was used.

The alkaline phenol and the caclium hypoch orite reagent were prepared according to the instructions given by FERRARI [4].

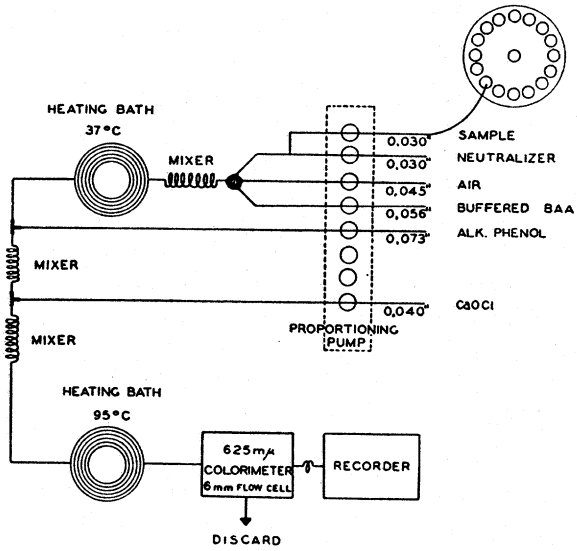

Fig. 1 — Flow chart and manifold components for the determination of amidasic activity.

EXPERIMENTAL PROCEDURE: The complete flow chart, with manifold components and tube sizes, is shown in Figure 1. The trypsin solution is automatically sampled, mixed with a buffered solution of BAA and permitted to circulate in a 40 feet long glass coil maintained at 37°C. During this incubation period the substrate is hydrolyzed by trypsin with the liberation of ammonia which reacts with the alkaline phenol and hypochlorite reagents when heated to 95°C giving a deep blue colour. The concentration of ammonia present is proportional to the colour formed and is determined with a flow colorimeter equipped with a 625 mμ filter and a 6 mm flow cell.

The method obtained above can be used for the determination of trypsin at the rate of 40 samples per hour.

DETERMINATION OF THE STERASIC ACTIVITY

Reagents: 1) TAME (p-tosyl-L-arginine methyl ester HCl) purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, was dissolved in Borate/HCl buffer (0.1M) of pH 8,5 to give a solution containing 14 mg per milliliter. 2) Sodium sulphite, 5% aqueous. 3) Potassium permanganate, 1% in aqueous 2% (v/v) sulphuric acid. 4) Two grams of chromotropic acid (1,8-dihydroxy-naphtalene-3,6-disulfonic acid, P5425. *The Matheson Co. Inc.*, East Rutherford, N.J.) were dissolved in 100 ml of water and filtered. To this filtered solution it was added sufficient 13.3M sulphuric acid to make one liter.

Experimental procedure: The complete flow chart with manifold components and tube sizes is shown in Figure 2.

The trypsin solution is automatically sampled and mixed with the buffered solution of TAME and permitted to circulate in a double mixing coil immersed in a constant temperature bath maintained at 37°C. During this incubation period, trypsin liberates methyl alcohol which is oxidized to formaldehyde by the acidic potassium permanganate solution. The permanganate is decolorized by the sulfite solution and the formaldehyde is then allowed to react with the chromotropic acid. This reaction takes place in a double coil heating bath maintained at 95°C. The developed colour is measured in a flow cell of 6 mm light path, using interference filters with peak transmission at 570 mμ.

concentration giving a zero order reaction; however, in order to save substrate all the other experiments were performed with a 2×10^{-3}M BAA solution.

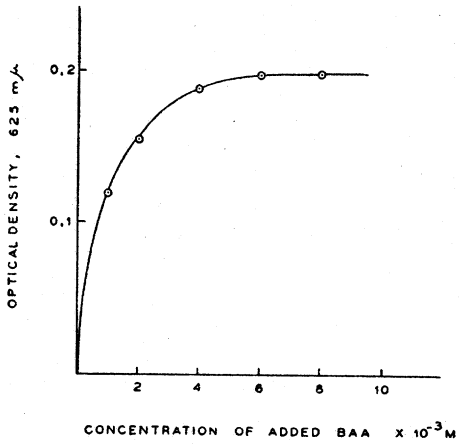

Fig. 4 — The effect of BAA concentration on the reaction velocity.

If the data from Figure 4 is plotted according with the method of LINEWEAVER AND BURK [10] a straight line is obtained and the Michaelis constant (Km) is found to be $2{,}5 \times 10^{-3}$M.

A very sensitive method for the determination of tryptic activity is achieved when TAME is used as the substrate. Figure 5 presents the correlation between trypsin concentration and the colour obtained by reaction with chromotropic acid. A straight line was obtained for trypsin concentrations from about 0,1 μg to 10 μg. This method is approximately 90 times more sensitive than the one resulting from the use of BAA as a substrate. The use of TAME as a substrate for trypsin presents other advantages which were pointed out by SCHWERT ET AL., i.e., it does not undergo spontaneous hydrolysis over a wide pH range; it is readily soluble in water; it is not hydrolyzed by chymotrypsin; and the products are not competitively inhibitory [6, 7, 8].

The flow chart of Figure 2, directly or with minor modifications, can be also used for the automatic determination of methyl alcohol and formaldehyde. For the determination of methyl alcohol the 0,030" tubing used for the buffered substrate and the first double mixing coil can be eliminated. In this case a sampling tube of 0,040" or samaller should be used. In the determination of formaldehyde all 0,030" tubing, the two double mixing coil and the first mixing coil can be supressed and the sampling performed by a 0,056" or smaller tubing. In any modification of the flow chart of Figure 2 it is always necessary to have in mind that the final concentration of sulfuric acid in the reaction mixture should be between 9 to 10M [11].

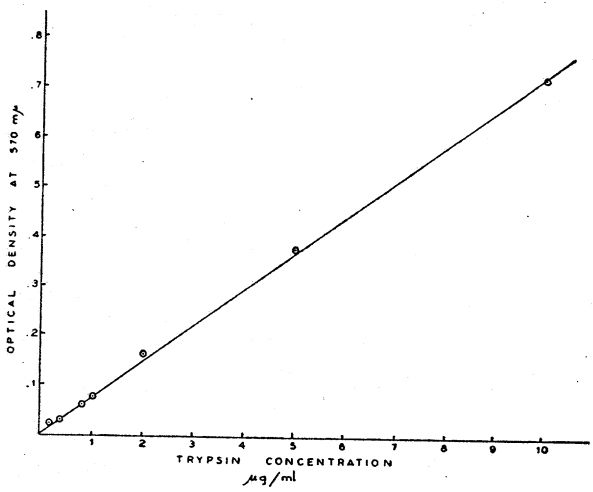

Fig. 5 — Calibration curve for trypsin esterasic activity.

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